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NF-κB-Inducing Kinase Establishes Self-Tolerance in a Thymic Stroma-Dependent Manner

Fumiko Kajitura,* Shijie Sun,* Takashi Nomura,‡ Keisuke Izumi,† Tomoo Ueno,§ Yoshimi Bando,‡ Noriyuki Kuroda,* Hongwei Han,* Yi Li,* Akemi Matsushima,* Yousuke Takahama,§ Shimon Sakaguchi,*¶ Tasuku Mitani,* and Mitsuru Matsumoto*‡

Physical contact between thymocytes and the thymic stroma is essential for T cell maturation and shapes the T cell repertoire in the periphery. Stromal elements that control these processes still remain elusive. We used a mouse strain with mutant NF-κB-inducing kinase (NIK) to examine the mechanisms underlying the breakdown of self-tolerance. This NIK-mutant strain manifests autoimmunity and disorganized thymic structure with abnormal expression of Rel proteins in the stroma. Production of immunoregulatory T cells that control autoreactive T cells was impaired in NIK-mutant mice. The autoimmune disease seen in NIK-mutant mice was reproduced in athymic nude mice by grafting embryonic thymus from NIK-mutant mice, and this was rescued by supply of exogenous immunoregulatory T cells. Impaired production of immunoregulatory T cells by thymic stroma without normal NIK was associated with altered expression of peripheral tissue-restricted Ags, suggesting an essential role of NIK in the thymic microenvironment in the establishment of central tolerance. 


A

utoimmune disease is a pathological condition in which the immune system turns on itself and causes serious damage to the organism’s tissues by as yet unknown mechanisms (1). A unifying concept for the mechanisms underlying the development of autoimmune disease has been one of the major challenges of immunological studies. Breakdown of self-tolerance is considered to be the key event for the disease process, and understanding the pathogenesis of this process is crucial for developing a therapeutic approach to these diseases. Because the thymus is the primary lymphoid organ for the establishment of self-tolerance, it is important to investigate how this process is controlled by the organization of the thymic microenvironment.

NF-κB-inducing kinase (NIK) is structurally related to mitogen-activated protein kinase kinase kinase (2) and has been shown to phosphorylate both Iκb kinase (IKK)-α and IKK-β, which sequentially activate the downstream Iκb proteins necessary for NF-κb activation (3, 4). The alymphoplasia (aly) strain of mice carries a natural mutation of the NIK gene (5, 6) in which a G855R substitution in the C terminus of the protein results in inability to bind to IKK-α (7). NIKalyaly mice have provided a unique model for the abnormal development of lymphoid organs; NIKalyaly mice lack all lymph nodes and Peyer’s patches, and spleen architecture such as development of germinal centers and follicular dendritic cell clusters is disturbed (5, 6, 8). We and others have demonstrated that this is due to defective NF-κB activation through the lymphotoxin (LT)-β receptor (LTβR) (6, 7, 9), a receptor essential for the development of lymphoid organs (10). Thymic structure is also disorganized in NIKalyaly mice, which is not observed in mice deficient for LT-α or LT-β (10); the medulla in NIKalyaly mice is smaller than that in NIKalyaly mice, and the boundary of the cortex and medulla is unclear (5, 6, 11). In addition to this abnormal lymphorganogenesis, NIKalyaly mice also serve as a model of autoimmune disease, but of unknown etiology (12); histopathological analysis of NIKalyaly mice has revealed chronic inflammatory changes in several organs, including the liver, pancreas, lung, salivary gland, and lacrimal gland (Refs. 5 and 12 and K. Izumi, Y. Bando, and M. Matsumoto, unpublished observation). Furthermore, these inflammatory changes in exocrine organs could be transferred into recombination-activating gene 2-deficient mice by a T cell-enriched fraction of spleen cells from NIKalyaly mice (12). We reasoned that the autoimmune disease phenotype seen in NIKalyaly mice might be associated with the altered thymic microenvironment.

One important role of the thymic stroma in establishing self-tolerance is the elimination of pathogenic autoreactive T cells by negative selection (13, 14). For this purpose, thymic epithelial cells need to express a set of self-Ags encompassing all the self-Ags expressed by parenchymal organs. Supporting this hypothesis, analysis of gene expression in thymic stroma has demonstrated that epithelial cells of the medulla are a specialized cell type in which promiscuous expression of a broad range of peripheral tissue-restricted genes is an autonomous property (15). Consistent with this notion, an autoimmune regulator (AIR) in thymic epithelial cells, a putative transcription factor, has been demonstrated...
to regulate this process (16), and deficiency of AIRE both in humans and in animal models results in the development of organ-specific autoimmune disease (16–18).

In addition to negative selection, self-tolerance is maintained by another mechanism involving immunoregulatory T cells. CD4+CD25+ T cells are immunoregulatory T cells that prevent CD4+ T cell-mediated organ-specific autoimmune diseases (19–22). The significance of this cell type in the maintenance of self-tolerance has been demonstrated by the fact that elimination of CD4+CD25+ T cells leads to the development of organ-specific autoimmune diseases in otherwise normal mice (23). Although it has been demonstrated that immunoregulatory T cells are substantially reduced in mice deficient in B7, CD28 (24), or CD40 (25), and that Foxp3, a transcription factor genetically defective in an autoimmune disease termed immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, is a key regulator for autoimmune diseases in otherwise normal mice (23). Although it was cultured for 4 days on top of Nucleopore filters (Whatman, Clifton, NJ) placed on RPMI 1640 medium (Invitrogen, San Diego, CA) supplemented with 10% heat-inactivated FBS (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-ME, hereafter referred to as R10, containing 1.35 mM 2′-deoxyguanosine (2-DG) (Sigma-Aldrich, St. Louis, MO). Five pieces of thymic lobes were grafted under the renal capsule of BALB/cnu/nu mice. After 6–8 wk, reconstitution of peripheral T cells was determined by flow cytometric analysis (BD Biosciences, Mountain View, CA) with anti-CD4 (clone GK1.5; BD Pharmingen, San Diego, CA) and anti-CD8 (clone 53-6.7; BD Pharmingen) mAbs, and then thymic chimeras were used for the analyses. In some cases, mice were injected with CD4+ T cells or CD4+CD25− T cells (2 × 103 cells per mouse) isolated from BALB/cnu/nu mice just after thymus graft.

Bone marrow (BM) transfer

BM transfer was performed as described previously (8). In brief, BM cells were suspended in R10 medium containing anti-CD90 (Thy1.2) mAb (clone 5a-8; Cedarlane Laboratories, Ontario, Canada) plus low toxicity rabbit C (Cedarlane Laboratories). After incubation at 37°C for 45 min, the cells were washed twice and adjusted to 3 × 106 viable cells/ml in R10. Each recipient mouse was lethally irradiated (10 Gy) and treated with 0.5 ml of donor BM cells i.v. on the same day. The recipient mice were used in the analyses 6–10 wk after BM transfer.

Western blotting

Proteins extracted from embryonic thymic lobes prepared as described above were analyzed using an ECL Western blotting detection system (Amersham, Piscataway, NJ). The Abs used were rabbit antipeptide Abs directed against p50 (cat. no. sc-114), p52 (cat. no. sc-298), ReA (cat. no. sc-109), ReB (cat. no. sc-226), and c-Rel (cat. no. sc-71), all purchased from Santa Cruz Biotechnology (Santa Cruz, CA) (8).

Pathology

Formalin-fixed tissue sections were subjected to H&E staining, and two pathologists independently evaluated the histology without being informed of the detailed condition of the individual mouse. Histological changes were scored as 0 (no change), 1 (mild lymphoid cell infiltration), or 2 (marked lymphoid cell infiltration).

Isolation and functional analysis of immunoregulatory T cells

Spleen cell suspensions were stained with FITC-conjugated anti-CD25 ( clone 7D4) and PE-conjugated anti-CD4 (clone H129.19) (BD Pharmingen), and sorted by a FACS (ALTRA; Beckman Coulter, Fullerton, CA) as previously described (23). Purity of the CD25+ and CD25+CD4+ populations was >90% and 95%, respectively. Spleen cells sorted as described above were cocultured with RBC-lysed and irradiated (15 Gy) spleen cells (5 × 107 from NIK+/+ mice as APCs for 3 days in 96-well round-bottom plates in R10. Anti-CD3 mAb (clone 145-2C11) (Cedarlane Laboratories) at a final concentration of 10 µg/ml was added to the culture for stimulation, and [3H] incorporation during the last 6 h of the culture was measured. For inoculation into thymus-grafted BALB/cnu/nu mice, CD4 T cells were isolated from spleen and lymph node of BALB/cnu/nu mice by using MACS CD4 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), as described previously (8). CD4+CD25− T cells were prepared by the depletion of CD25+ T cells with anti-CD25 Ab plus low toxicity rabbit C.

Real-time PCR

Real-time PCR for the quantification of Foxp3 was conducted with cDNA prepared from RNA extracted from CD4+ splenocytes and whole thymus. The primers and the probe used were as previously described (26). Real-time PCR were performed in a final volume of 20 µl with 400 nM of the forward and reverse primers and 200 nM of the probe by use of a QuantiTect Probe PCR Kit (Qiagen, Valencia, CA). Reactions were run on an ABI/PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA) in triplicate. Cycling conditions were a single denaturing step at 95°C for 15 min followed by 45 cycles of 94°C for 15 s and 60°C for 1 min.

Semiquantitative RT-PCR

RNA was extracted from whole thymus with TRIzol (Invitrogen), and treated with DNase to eliminate any contaminating DNA. After phenol/chloroform extraction and ethanol precipitation, 5 µg of total RNA were

**Materials and Methods**

**Mice**

NIK+/+ mice, NIK+/− mice, and BALB/cJel-μ mice (BALB/cnu/nu mice) were purchased from CLEA Japan (Osaka, Japan). LT−/− mice are the generous gift from Dr. D. D. Chaplin (University of Alabama, Birmingham, AL). The mice were maintained under pathogen-free conditions, and were handled in accordance with the Guidelines for Animal Experimentation of Tokushima University, School of Medicine (Tokushima, Japan). The experiments were initiated at 8–12 wk of age.

**Immunohistochemistry**

Immunohistochemical analysis of the thymus was performed as previously described (8, 9). Briefly, frozen tissue sections were fixed in cold acetone, and stained by first incubating with peanut agglutinin (PNA)-biotin and Ulex europaeus agglutinin 1 (UEA-1)-biotin (Vector Laboratories, Burlingame, CA). After being washed, the sections were further incubated with streptavidin conjugated with alkaline phosphatase (AP) (Zymed Laboratories, San Francisco, CA). Color development for bound AP was with an AP reaction kit (Vector Laboratories). For the confocal microscopic analysis, Alexa 488 (Molecular Probes, Eugene, OR) -conjugated anti-mouse CD4 (clone GK1.5), Alexa 546-conjugated CD8 (clone 53-6.7) and ER-TR5 mAbs were used. Alexa 633-conjugated anti-rat IgG (Molecular Probes) was used for the detection of ER-TR5 binding. For the detection of anti-nuclear Ab (ANA), serum from thymic chimeras was incubated with HepG2 cells grown on glass slides. FITC-conjugated anti-mouse IgG Ab (Southern Biotechnology Associates, Birmingham, AL) was used for the detection.
subjected to oligo(dT)-primed reverse transcription with a cDNA Cycle kit (Invitrogen). The primer pairs used for PCR were as previously described (16). PCR was conducted in a final volume of 30 μl with 1.5 U of ExTaq DNA polymerase (Takara Biomedicals, Otsu, Japan) and 250 nM of each primer. Cycling conditions were a single denaturing step at 94°C for 3 min followed by either 35 cycles (for tissue-specific Ags) or 25 cycles (for β-actin) of 94°C for 45 s, 50–54°C for 45 s, and 72°C for 1 min, followed by a final extension step of 72°C for 3 min. For AIRE, a single denaturing step at 95°C for 3 min was followed by 35–40 cycles of 95°C for 30 s and 65°C for 1 min.

**Results**

*NIK in the thymic stroma is required for self-tolerance*

Thymic structure is disorganized in NIK<sup>aly/aly</sup> mice; the boundary of the cortex and medulla revealed with H&E staining is unclear, and the medulla contained fewer epithelial cell components compared with that from NIK<sup>aly/+</sup> mice (Fig. 1Aa). We then used immunohistochemistry to investigate thymic organization in NIK<sup>aly/aly</sup> mice. The medulla in NIK<sup>aly/aly</sup> mice was smaller than that in NIK<sup>aly/+</sup> mice, and

**FIGURE 1.** NIK mutation affects thymic organization. *A*, H&E staining revealed that medulla from NIK<sup>aly/aly</sup> mice (a, right panel) contained fewer epithelial cells compared with that from NIK<sup>aly/+</sup> mice (a, left panel). Arrows indicate the boundaries between the cortex and the medulla. ER-TR5-positive medullary cells (stained in orange) are sparse in the NIK<sup>aly/aly</sup> thymus (b, right panel) compared with that from NIK<sup>aly/+</sup> mice (b, left panel). Thymocytes are stained with anti-CD4 and anti-CD8 Abs. Areas of medulla are identified as CD4<sup>+</sup> T cell-rich areas (stained green), whereas areas of cortex are composed mainly of CD4<sup>+</sup>CD8<sup>+</sup> T cells (stained light blue). Thymic medullas, identified as the PNA-negative area, from NIK<sup>aly/aly</sup> mice contain no UEA-1<sup>+</sup> cells (c, right panel) (stained in blue in NIK<sup>aly/+</sup> mice; c, left). Thymocytes in the cortex are stained with PNA also in blue. Embryonic thymus from NIK<sup>aly/aly</sup> mice contained UEA-1<sup>+</sup> cells in the medulla (PNA-negative area) after grafting onto BALB/c<sup>nu/nu</sup> mice (d, left panel), whereas embryonic thymus from NIK<sup>aly/+</sup> mice did not acquire UEA-1<sup>+</sup> cells after the same treatment (d, right panel). Staining is the same as in c. Asterisks represent the kidney tissues adjacent to the grafted thymus. Scale bars correspond to 100 μm in size. *B*, Thymic lobes isolated from NIK<sup>aly/+</sup> embryos and cultured for 4 days in the presence of 2-DG contain no live thymocytes as determined by flow cytometric analysis (right panels). Cells of FSC>58 were excluded from the analysis. In contrast, thymic lobes cultured in the absence of 2-DG supported maturation of thymocytes (left panels). FSC, forward scatter; SSC, side scatter. *C*, Absence of live thymocytes in embryonic thymus cultured with 2-DG was demonstrated by the lack of Ick expression with Western blotting (top). The same blot was probed with anti-Rel proteins. p52 processing from a precursor p100 was impaired in thymic stroma (+2DG) as well as in whole thymic lobes (−2DG) from NIK<sup>aly/aly</sup> mice. RelB expression was also reduced in NIK<sup>aly/aly</sup> mice. Total splenocytes (Spl) from wild-type mice were used as control.
ER-TR5-positive medullary epithelial cells were sparse (Fig. 1Ab). Because thymocytes in the cortex bind with PNA (29), the medulla can be identified as a PNA-negative area. We found that medullary epithelial cells that bind with the lectin UEA-1 (30) were absent from can be identified as a PNA-negative area. We found that medullary epithelial cells in the cortex bind with PNA (29), the medulla can be identified as a PNA-negative area. We found that medullary epithelial cells that bind with the lectin UEA-1 (30) were absent from

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Although reduced in number, CD4+CD25+ T cells isolated from NIK<sup>aly/aly</sup> mice dose-dependently suppressed [3H]thymidine uptake by native T cells cocultured in vitro with an efficiency nearly identical to that of CD4+CD25+ cells from NIK<sup>aly/aly</sup> mice (Fig. 3B); addition of IL-2 abolished the suppressive function of immunoregulatory T cells in both cases. Thus, although NIK plays an important role in the production of immunoregulatory T cells, NIK is not required for the suppressive function of this cell type. Together with the results from thymic chimeras, these results suggest that NIK plays an important role in the establishment of self-tolerance, in part through the generation of the thymic microenvironment that controls the production of immunoregulatory T cells.

**Rescue of self-tolerance by immunoregulatory T cells**

We investigated whether the autoimmune-disease phenotype observed in BALB/c<sup>nu/nu</sup> mice grafted with NIK<sup>aly/aly</sup> embryonic thymus (right panels), but not with NIK<sup>aly/aly</sup> embryonic thymus (left panels), developed an autoimmune-disease phenotype in the liver (a) and pancreas (b). Arrows indicate the lymphoid cell infiltrations. A scale bar corresponds to 100 μm in size. IgG class ANA were detected in serum from BALB/c<sup>nu/nu</sup> mice grafted with NIK<sup>aly/aly</sup> embryonic thymus (c, right). Negative ANA from NIK<sup>aly/aly</sup> thymic chimera is shown on the left. Original magnification, ×100. B. Elevation of serum transaminases were observed from BALB/c<sup>nu/nu</sup> mice grafted with NIK<sup>aly/aly</sup> embryonic thymus (n = 8), but not from BALB/c<sup>nu/nu</sup> mice grafted with NIK<sup>aly/aly</sup> embryonic thymus (n = 5). Each circle corresponds to one mouse. C, Many NIK<sup>aly/aly</sup> thymus-grafted BALB/c<sup>nu/nu</sup> mice exhibited lymphoid cell infiltrations in the liver (top panel) and pancreas (bottom panel). In contrast, these changes were scarcely observed in NIK<sup>aly/+/</sup> thymus-grafted mice. BALB/c<sup>nu/nu</sup> mice grafted with both NIK<sup>aly/+/</sup> thymus and NIK<sup>aly/aly</sup> thymus showed intermediate changes.

Injection of CD4<sup>+</sup> T cells into NIK<sup>aly/+/</sup> thymus-grafted BALB/c<sup>nu/nu</sup> mice (2 × 10<sup>7</sup> cells per mouse) completely rescued the inflammatory changes, and CD4<sup>+</sup>CD25<sup>+</sup>T cells showed a modest effect. Histological changes in H&E-stained tissue sections were scored as 0 (no change), 1 (mild lymphoid cell infiltration), or 2 (marked lymphoid cell infiltration). One mark corresponds to one mouse analyzed. D, NIK<sup>aly/aly</sup> mice reconstituted with NIK<sup>aly/+/</sup> BM displayed marked inflammatory lesions in pancreas and salivary gland, whereas NIK<sup>aly/+/</sup> mice reconstituted with NIK<sup>aly/aly</sup> BM showed few such inflammatory changes.
FIGURE 3. NIK regulates production of immunoregulatory T cells. A, Spleens from NIK<sup>aly/aly</sup> mice (■) contained reduced percentages as well as total numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells compared with those from NIK<sup>wt</sup> mice (□) (top panel); n = 6, p < 0.01. Reduced numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cells in NIK<sup>aly/aly</sup> mice were also observed in the thymus (bottom panel); n = 6, p < 0.05. B, CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from NIK<sup>aly/aly</sup> mice (■) dose-dependently suppressed <sup>3</sup>H]thymidine uptake by native T cells cocultured in vitro with an efficiency nearly identical to that of CD4<sup>+</sup>CD25<sup>+</sup> cells from NIK<sup>wt</sup> mice (□). One representative result from a total of three repeats is shown.

NIK mutation is associated with altered gene expression in the thymus

The mechanism that controls the thymic microenvironment in a NIK-dependent fashion is of considerable interest. We wanted to determine the gene(s) relevant to the role of the thymic stroma in the development of the autoimmune-disease phenotype in NIK<sup>aly/aly</sup> mice. One candidate gene is AIRE, mutation of which is responsible for the autoimmune-polyendocrinopathy-candidiasis ectodermal dysplasia syndrome, which shows monogenic autosomal recessive inheritance (18). Because AIRE is strongly expressed in medullary epithelial cells in the thymus (15, 16, 18), we suspected that expression of AIRE in the thymus might be changed in the absence of normal NIK. Therefore, we prepared RNA from whole thymus and performed RT-PCR for AIRE. AIRE expression in NIK<sup>aly/aly</sup> thymus was dramatically reduced compared with that from NIK<sup>wt</sup> thymus (Fig. 4A, left), Top). Real-time PCR demonstrated that AIRE expression level from NIK<sup>aly/aly</sup> thymus was 1–5% of that from NIK<sup>aly/aly</sup> thymus (N. Kuroda, H. Han, and M. Matsumoto, unpublished observation).

"Aberrant" or "promiscuous" expression of a broad range of peripheral tissue-restricted genes by thymic epithelial cells has been implicated in the essential process of establishing self-tolerance (15), and AIRE in thymic epithelial cells has been demonstrated to control this promiscuous gene expression (16). We used semiquantitative RT-PCR to investigate the expression of peripheral tissue-restricted genes in RNAs extracted from the whole thymus. The NIK<sup>aly/aly</sup> thymus showed easily detectable expression of tissue-specific Ags, including salivary protein 1, fatty acid binding protein, glutamate decarboxylase 67, and C-reactive protein (Fig. 4A, left). In contrast, those messages were hardly, if at all, detected in NIK<sup>aly/aly</sup> thymus. Thus, tissue-specific Ag expression was dramatically reduced in NIK<sup>aly/aly</sup> thymus. As expected from normal thymic structure in LT-α<sup>−/−</sup> mice, LT-α<sup>−/−</sup> thymus demonstrated indistinguishable levels of AIRE and tissue-specific Ag expression from those of control thymus (Fig. 4A, right).

Although AIRE has been proposed to regulate promiscuous gene expression of many peripheral tissue-restricted Ags in the thymus (16), it is not clear whether the promiscuity in gene expression is confined to AIRE-expressing cells. It is possible that many tissue-specific Ags are also expressed in medullary epithelial cells which do not express AIRE, and NIK may affect the development of such cell types as well, resulting in the dramatic reduction of tissue-specific Ag expression. To investigate this, we first adjusted the amount of cDNA to normalize the AIRE expression level between NIK<sup>aly/aly</sup> thymus and NIK<sup>aly/aly</sup> thymus (Fig. 4B). In this experimental setting, we detected much stronger housekeeping gene (β-actin) expression from...
NIK<sup>aly/aly</sup> thymus cDNA compared with NIK<sup>aly/+</sup> thymus cDNA (Fig. 4B). Given that AIRE is predominantly expressed by the thymic medulla (16, 18) (from both UEA-1<sup>+</sup> and UEA-1<sup>+</sup> cells) (15), this result supports that AIRE-expressing medullary epithelial cells are fewer in NIK<sup>aly/aly</sup> thymus compared with NIK<sup>aly/+</sup> thymus. We then tested the expression of peripheral tissue-restricted Ags by these AIRE-normalized cDNA samples, which should represent RNAs derived from roughly equal amounts of AIRE-expressing cells from both NIK<sup>aly/aly</sup> and NIK<sup>aly/+</sup> thymus. Expression of peripheral tissue-restricted Ags was still weaker in NIK<sup>aly/aly</sup> thymus compared with NIK<sup>aly/+</sup> thymus (Fig. 4B); by real-time PCR, the salivary protein 1 expression level from NIK<sup>aly/aly</sup> thymus was less than one third that of NIK<sup>aly/+</sup> thymus when the AIRE expression level was normalized (N. Kuroda, H. Han, and M. Matsumoto, unpublished observation). These results suggest that reduced AIRE expression in NIK<sup>aly/aly</sup> thymus alone does not fully account for the reduced expression of peripheral tissue-restricted Ags. Rather, our results suggest the existence of AIRE-negative thymic epithelial cells which equally play important roles in the expression of peripheral tissue-restricted Ags; development of such cell types is defective in NIK<sup>aly/aly</sup> mice, as observed for the development of AIRE-expressing cells.

**Discussion**

We have demonstrated that NIK plays an essential role in the organization of the thymic microenvironment that is required for the establishment of self-tolerance. The breakdown of self-tolerance in the absence of normal NIK in thymic stroma involved the processes for the production of immunoregulatory T cells, and the defect was rescued by the exogenous supply of immunoregulatory T cells. NIK was originally identified as a kinase required for NF-κB activation induced by a wide variety of ligand binding (2). It is now clear, however, that the requirement for NIK for NF-κB activation is strictly signal-dependent; NF-κB activation induced by TNF-α takes place without NIK, whereas NIK is essential for NF-κB activation downstream of the LTβR (7, 33). The NIK-related signaling pathway(s) involved in the establishment of self-tolerance in a thymic-stroma dependent fashion are presently unknown. Because LT-α/− mice possess normal thymic architecture with normal expression of AIRE and peripheral tissue-Ags, and grafting of the embryonic thymus from LT-α/− mice (which had been developed in the absence of LT in its early ontogeny) onto BALB/c<sup>nu/nu</sup> mice did not induce any inflammatory changes in the liver and pancreas of recipient mice (F. Kajiura, K. Izumi, Y. Bando, and M. Matsumoto, unpublished observation), it is reasonable to speculate that membrane-bound LT is not solely responsible for this action. Given that signals through LTβR control thymic organogenesis (31), LIGHT and/or other unidentified LTβR ligands(s) might be responsible for the phenotypes described in the present study. However, it remains also possible that NIK is acting downstream of other receptor(s) beyond LTβR in this process. Of note, the pathway(s) regulate the processes involved in the generation of p52 from a precursor p100 (Fig. 1C), as observed for signals through LTβR (34–37).

We have established that NIK is required for the production of immunoregulatory T cells; this was demonstrated both by the reduction of CD4<sup>+</sup>CD25<sup>+</sup> T cell numbers and by the reduced expression of Foxp3, a newly identified functional marker for immunoregulatory T cells (26–28). Supporting this, an exogenous supply of sufficient numbers of immunoregulatory T cells was able to rescue the autoimmune disease phenotype not only in NIK<sup>aly/aly</sup> thymus-grafted BALB/c<sup>nu/nu</sup> mice (Fig. 2C), but also in NIK<sup>aly/aly</sup> mice themselves (M. Minami, M. Nakazawa, and C. Tamura, personal communication). It will be important to study the nature of the TCR ligands and the mechanisms involved in the NIK-dependent production of immunoregulatory T cells. It has been proposed that immunoregulatory T cells may arise from relatively high avidity interactions with self-peptide-MHC complexes just below the threshold for negative selection in the thymus (38, 39). Accordingly, thymic stroma which have been developed in the absence of normal NIK may not be able to present TCR ligands (most likely containing self-peptides) efficiently enough, resulting in insufficient avidity for the production of immunoregulatory T cells.

Given that alteration in the pattern of gene promiscuity in the thymic stroma affects the production of immunoregulatory T cells, the process for negative selection might also be affected in NIK<sup>aly/aly</sup> mice. We have tested this possibility by the use of a TCR transgenic model specific for male H-Y Ag (14). Unexpectedly, elimination of autoreactive T cells clonotypic for H-Y TCR in the male was not obviously changed in NIK<sup>aly/aly</sup> mice. We have tested the possibility by the use of a TCR transgenic model specific for male H-Y Ag (14). Unexpectedly, elimination of autoreactive T cells clonotypic for H-Y TCR in the male was not obviously changed in NIK<sup>aly/aly</sup> mice. We have tested the possibility by the use of a TCR transgenic model specific for male H-Y Ag (14). Unexpectedly, elimination of autoreactive T cells clonotypic for H-Y TCR in the male was not obviously changed in NIK<sup>aly/aly</sup> mice. We have tested the possibility by the use of a TCR transgenic model specific for male H-Y Ag (14). Unexpectedly, elimination of autoreactive T cells clonotypic for H-Y TCR in the male was not obviously changed in NIK<sup>aly/aly</sup> mice. We have tested the possibility by the use of a TCR transgenic model specific for male H-Y Ag (14). Unexpectedly, elimination of autoreactive T cells clonotypic for H-Y TCR in the male was not obviously changed in NIK<sup>aly/aly</sup> mice. We have tested the possibility by the use of a TCR transgenic model specific for male H-Y Ag (14). Unexpectedly, elimination of autoreactive T cells clonotypic for H-Y TCR in the male was not obviously changed in NIK<sup>aly/aly</sup> mice. We have tested the possibility by the use of a TCR transgenic model specific for male H-Y Ag (14). Unexpectedly, elimination of autoreactive T cells clonotypic for H-Y TCR in the male was not obviously changed in NIK<sup>aly/aly</sup> mice. We have tested the possibility by the use of a TCR transgenic model specific for male H-Y Ag (14). Unexpectedly, elimination of autoreactive T cells clonotypic for H-Y TCR in the male was not obviously changed in NIK<sup>aly/aly</sup> mice. We have tested the possibility by the use of a TCR transgenic model specific for male H-Y Ag (14). Unexpectedly, elimination of autoreactive T cells clonotypic for H-Y TCR in the male was not obviously changed in NIK<sup>aly/aly</sup> mice. We have tested the possibility by the use of a TCR transgenic model specific for male H-Y Ag (14). Unexpectedly, elimination of autoreactive T cells clonotypic for H-Y TCR in the male was not obviously changed in NIK<sup>aly/aly</sup> mice.
simultaneously onto BALB/cnu/cnu mice, the development of inflammatory lesions was not completely inhibited (Fig. 2C). This result suggests that impaired production of immunoregulatory T cells is not the only defect, but is accompanied by the existence of a dominant process for the breakdown of self-tolerance determined by the thymic stroma without normal NIK. In this scenario, it is possible that the grafted NIK^aly/aly thymus allows production of more pathogenic autoreactive T cells in the recipient mice than can be controlled by the immunoregulatory T cells that are produced by the grafted NIK^{+/+} thymus.

Although the exact mechanism by which NIK regulates the thymic microenvironment that is required for the establishment of central tolerance is unknown, the disorganized thymic structure, together with reduced AIRE expression in mice with a mutation disrupting the RelB gene merits attention (40, 41). Because of the phenotypic similarities between NIK^aly/aly and RelB^-/- mice (42) (multi-inflammatory lesions together with the absence of UEA-1^-/- medullary epithelial cells in the thymus), we speculate that NIK regulates the thymic microenvironment through the activation of the NF-κB complex containing RelB. Based on the findings that production of p52 is impaired in NIK^aly/aly thymic stroma (Fig. 1C) and that NIK has been demonstrated necessary for the production of NK T cells through the action of RelB (43, 44), we further speculate that the NIK-related signaling pathway(s) activate the NF-κB complex in thymic stroma mainly consisting of p52/RelB heterodimers to generate the thymic microenvironment that is necessary for the establishment of self-tolerance.

We have demonstrated that AIRE expression is significantly reduced in NIK^aly/aly thymus. Although we suggest that a developmental effect of mutated NIK on thymic epithelial cells expressing AIRE (i.e., fewer medullary epithelial cells) seems to be the likely explanation, we cannot exclude the possibility that reduced AIRE expression in NIK^aly/aly thymus is due to down-regulation of AIRE by the mutated NIK through a transcriptional mechanism. The latter possibility needs to be tested in future work.

Promiscuous gene expression of many peripheral tissue-restricted Ags in the thymic epithelial cells play essential roles in the establishment of self-tolerance, and AIRE has been proposed to be essential in this process (15, 16). We have demonstrated that promiscuous gene expression is dramatically reduced in the NIK^aly/aly thymus, and we speculate that developmental effect of NIK on thymic epithelial cell components (including both AIRE-expressing cells and AIRE-negative cells) is responsible for the reduced gene expression of many peripheral tissue-restricted Ags in NIK^aly/aly thymus. In contrast, AIRE seems to regulate expression of peripheral tissue-restricted Ags predominantly through a transcriptional mechanism, because the thymic structure is apparently unaffected by the absence of AIRE (16, 17); this possibility might be consistent with the demonstration that AIRE can interact with CREB-binding protein/p300 (45). Thus, there must be a group of genes that together control promiscuous gene expression in the thymus through their unique actions, as exemplified by NIK and AIRE. Therefore, it is critical to determine whether the reduced promiscuous gene expression in the thymus and the development of autoimmune diseases are directly linked in future study.

Finally, although both impaired production of immunoregulatory T cells and possibly an impaired negative selection process contribute to the development of autoimmunity in NIK^aly/aly mice, the exogenous supply of sufficient numbers of immunoregulatory T cells was able to rescue the autoimmune disease phenotype, supporting the therapeutic benefits of immunoregulatory T cells. With the advent of thymic organogenesis using thymic precursor cells (46, 47), it may be feasible to manipulate the thymic microenvironment, thereby controlling the processes for the establishment of self-tolerance.

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